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Binding and hydrolysis of 2-azido-ATP and 8-azido-ATP by isolated mitochondrial F_1: characterisation of high-affinity binding sites

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The kinetic parameters for the hydrolysis by F_1 of the photoreactive nucleotide analogue 2-azido-ATP were determined (V_{max}, 105 U/mg F_1; K_m, 250 μM, in the presence of 1.0 mM SO_3^{2-}). In the absence of an activating anion, a non-linear relationship in a Lineweaver-Burk plot was found for the hydrolysis of 2-azido-ATP. The 2-azido-analogues of ATP and ADP proved to be good photoaffinity labels causing notable inactivation of the F_1-ATPase activity upon irradiation at 360 nm. This inhibition was also used to demonstrate high-affinity binding of these analogues to a catalytic binding site on the F_1. High-affinity binding proved to be an Mg^{2+}-requiring process, occurring with both 2-azido-ATP and 2-azido-ADP but hardly or not occurring with 8-azido-AT(D)P. Covalent binding of 2-nitreno-ATP upon irradiation of F_1 containing tightly bound [β-^{32}P]2-azido-ATP results in a proportional inhibition of ATPase activity, extrapolating to 0.92 mol of covalently bound label per mol of F_1 needed for the complete inactivation of the enzyme. When the F_1 was irradiated in the presence of excess [β-^{32}P]2-azido-AT(D)P, 3–4 mol of label were bound when the enzyme was fully inactivated. In all cases, all or most of the radioactivity was found on the β subunits.

Introduction

The F_1 part of the ATP synthase complex is composed of five different subunits in the following stoicheiometry α_3, β_3, γ, δ, ϵ [1]. It has been demonstrated that the six nucleotide binding sites present on the F_1 are located on the α and β subunits [2–4]. The nucleotide binding sites can be divided in catalytic and non-catalytic binding sites, the actual number of catalytic sites having been a matter of debate for a long time [2,5,6]. Evidence has accumulated that the catalytic sites are located on the β subunits [4,7], in agreement with the finding that most ATPase inhibitors interact with the β subunits.

Besides, isolated F_1 is known to contain 2 or 3 nucleotides tightly bound to the enzyme [2,3,8,9]. The function and the localisation of these tightly bound nucleotides is currently being investigated in our laboratory, the question being if and how they are involved in the reaction mechanism of ATP-hydrolysis and -synthesis. To investigate this, the photoreactive nucleotides 2-azido-AT(D)P and 8-azido-AT(D)P are used. With the 8-azido analogues, catalytic and non-catalytic binding sites could be demonstrated on the F_1, located on the β subunits and the interfaces between α and β subunits, respectively [7,10]. It was also shown that the tightly bound nucleotides cannot be exchanged with the 8-azido nucleotides [2,7,10]. As

Abbreviations: 8(2)-N-AT(D)P, 8(2)-nitrenoadenosine-5′-tri(di)-phosphate; Mes, 4-morpholineethanesulphonic acid; Mops, 4 morpholinepropanesulphonic acid.

the tightly bound nucleotides were shown to exchange very slowly with medium nucleotides [11], and as the function of the tight nucleotide binding sites appeared to be at most regulatory [12], they were not considered to be directly involved in catalysis. This is supported by experiments with nucleotide-depleted F_1 which could bind five molecules of [^{14}C]ATP per F_1, only two of which are exchangeable [3]. On the other hand, evidence is accumulating that tight binding may be an intermediary phase in the reaction mechanism of catalysis [5,7,13–17].

As at least two catalytic sites could be demonstrated on two β subunits with 8-azido-AT(D)P [2,7,10,18], it is theoretically possible that the third β subunit contains a tightly bound nucleotide involved in catalysis of conversion of substrates that can be bound tightly to the enzyme. It has been proposed [19] that for tight binding an 'anti' configuration of the bound nucleotide is required. This made it necessary to expand our studies with azido analogues in the 'anti' configuration. In the present paper, it is shown that the very hydrolysable 2-azido-ATP is also a valuable photoaffinity label which can be bound with high affinity, due to its 'anti' configuration. It is demonstrated that this tight binding to a catalytic site is an intermediate phase in the reaction mechanism.

Materials and Methods

Bovine heart mitochondrial F_1 was isolated according to Knowles and Penefsky [20] and stored in liquid nitrogen in a medium comprising 10 mM Tris-HCl (pH 7.5), 4 mM ATP, 250 mM sucrose and 4 mM EDTA. Prior to use, F_1 was subjected to ammonium sulphate precipitation and was subsequently filtered three times by centrifugation through a column (Penefsky column [21]) which contained Sephadex G-50 coarse pre-equilibrated in the same medium, except that ATP was omitted (medium A). In some of the experiments, the Sephadex used was pre-equilibrated in medium in which EDTA was replaced by 6 mM $MgCl_2$ (medium B). When optimal turnover conditions (ATP hydrolysis) were needed for the experiment, 10 mM HCO_3^- was added to medium B and the pH was raised to 8.

The photoaffinity labels 2-azido-ATP and 2-azido-ADP were synthesized in our laboratory; 2-chloroadenosine was mixed with anhydrous hydrazine under a nitrogen atmosphere at room temperature for 16 h according to the method of Schaefer and Thomas [22]. The 2-hydrazine-adenosine formed was isolated by evaporating the hydrazine and, after coevaporating residual hydrazine with added isopropanol, was subsequently solubilised in 5% acetic acid. After the addition of the sodium nitrite solution at 0°C, the 2-azido-adenosine started to crystallise after 10 min. The 2-azidoadenosine (75%) was isolated on a Büchner filter, washed with cold water and dried over phosphorpentoxide. Phosphorylation of 2-azido-adenosine was carried out as described by Sowa and Ouchi [23]. 2-Azidoadenosine 5′-monophosphate (60%) was isolated and purified on a DEAE-Sephadex A-25 column (50 × 2 cm) by eluting with a 0–300 mM triethylamine-HCO_3^- gradient (pH 7.8). Synthesis of 2-azido-ADP and 2-azido-ATP was carried out as described by Hoard and Ott [24], starting with a dry solution of 2-azido-AMP as a tributylamine salt in dimethylformamide. After purification on a DEAE-Sephadex A-25 column (50 × 2 cm) with 100–800 mM triethylamine-HCO_3^- gradient (pH 7.8), 50–60% of the purified product was solubilised in acetone ethanol. After addition of LiCl, the Li salt was centrifuged and solubilised in a 10 mM Tris-HCl solution (pH 7.0). The azido analogues were stored at −20°C. Spectral and analytical data were in agreement with those published by Czarnecki et al. [25]. [β-^{32}P]2-azido-ADP was synthesized in the same way, using 10 mCi [^{32}P]P_i (45 μmol). About 30% of the 2-azido-AMP (20 μmol) was transformed into the labelled compound which was phosphorylated in [β-^{32}P]2-azido-ATP using the phospho*enol*pyruvate/pyruvate kinase system. 8-Azido-AT(D)P and [2-^{3}H]8-azido-AT(D)P were synthesized as described previously [26].

ATP hydrolysis activity was measured spectrophotometrically by following the oxidation of NADH in an ATP-regenerating system as described by Sloothaak et al. [26]. For K_m determinations, the ATPase medium contained varying concentrations of MgATP, Mg-8-azido-ATP or Mg-2-azido-ATP. Furthermore, the pyruvate kinase concentration was raised 5-times and 10 mM HCO_3^- or 1 mM SO_3^{2-} was added as an

activating anion. ATP hydrolysis, as measured by phosphate production, was performed by taking samples from the F_1-containing reaction medium at several time intervals. The amount of phosphate produced was measured as described by Fiske and Subbarow [27].

Photoaffinity labelling was performed by irradiation at room temperature at 360 nm using a CAMAG Universal ultraviolet lamp. Irradiation of tightly incorporated 2-azido-nucleotides was performed on hollow sheets of aluminium. The total amount of covalently bound [β-^{32}P]2-N-AT(D)P was measured after heat denaturation of the F_1 (5 min, 95°C) and centrifugation by counting the radioactivity in the pellet. The distribution of radioactivity on α and β subunits was determined via SDS-urea polyacrylamide gel electrophoresis as described previously [7].

Protein concentrations were determined as described by Lowry et al. [28], using bovine serum albumin as a standard. ATP, phospho*enol*pyruvate, lactate dehydrogenase and NADH were purchased from Boehringer Mannheim. All other chemicals used were of analytical grade.

Results

Hydrolysis

The nucleotide analogue 2-azido-ATP can be considered as a better analogue of ATP than 8-azido-ATP. This is illustrated in Table I where K_m and V_{max} values are reported for the steady-state hydrolysis of ATP and the two photoreactive nucleotides 2-azido-ATP and 8-azido-ATP. It can be seen that the V_{max} value for the hydrolysis of 2-azido-ATP approaches a value much closer to that of ATP than the V_{max} value 8-azido-nucleotide. Furthermore, it has to be taken into account that the 2-azido-ATP is in equilibrium with tetrazolic iso-mers: under the conditions used (aqueous solution, pH 8.0) about 55% can be expected to be in one of these two tetrazolic forms [25]. The real K_m value for 2-azido-ATP, as reported in Table I, is, therefore, about 2.2-times lower than the measured apparent value.

In Fig. 1, it is shown that the steady-state hydrolysis of 2-azido-ATP in the absence of any activating anion showed negative cooperativity, just as the hydrolysis of ATP. The main difference

TABLE I

KINETIC PARAMETERS FOR THE STEADY-STATE HYDROLYSIS OF ATP, 2-AZIDO-ATP AND 8-AZIDO-ATP

K_m values of the hydrolysis of the different nucleotides were measured in an ATP-regenerating system (see Materials and Methods) in the presence of 10 mM HCO_3^- (ATP and 8-azido-ATP) or 1 mM SO_3^{2-} (2-azido-ATP). The K_m value for the hydrolysis of 2-azido-ATP has been corrected for the presence of 55% tetrazolic isomer [25]; the measured K_m was 545 μM.

Substrate	K_m (μM)	V_{max} (U/mg F_1)
ATP	118	160
2-azido-ATP	250	105
8-azido-ATP	500	14.8

is the V_{max}, ATP being hydrolysed 3-times faster than 2-azido-ATP. When the rate of hydrolysis of the nucleotides was followed by measuring the phosphate production (Fig. 2), it could be seen that only part of the 2-azido-ATP was rapidly hydrolysed, followed by a slow phase of hydrolysis. In this phase, the slow isomerisation between the tautomeric forms is likely to be the rate-limiting event if the tetrazolic form is not hydrolysed or does so only slowly. When 2-azido-ATP was added as a solution in methanol, in which more than 80% of the analogue is present in the azido form [25], the initial amount of phosphate pro-

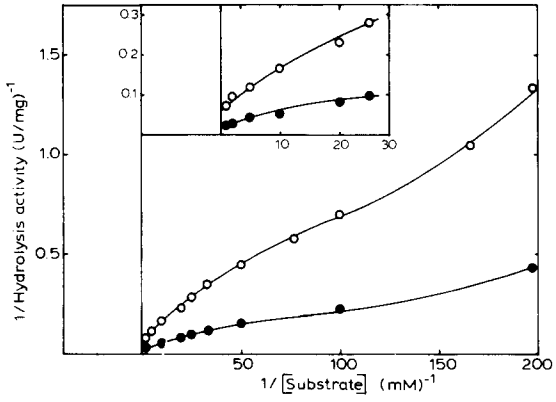

Fig. 1. Kinetics of ATP (●) and 2-azido-ATP (○) hydrolysis by isolated F_1-ATPase in the absence of activating anions. Measured kinetic parameters were: for 2-azido-ATP: V_{max}, 14.6 U/mg and K_m, 187 μM, for ATP: V_{max}, 50 U/mg and K_m, 265 μM. The K_m values were the values belonging to the higher nucleotide concentrations.

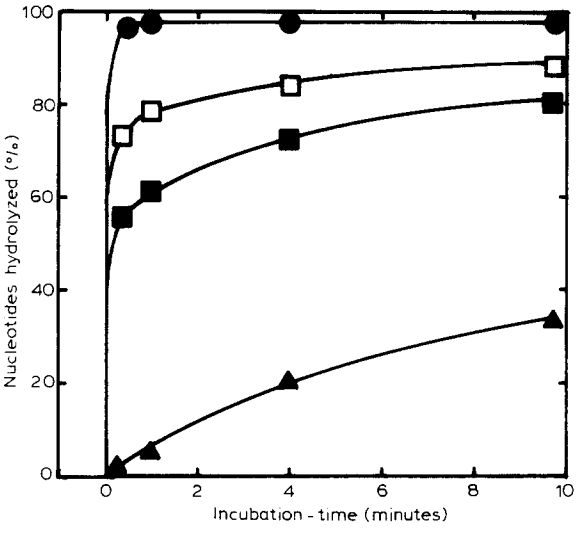

Fig. 2. Time-dependent phoshate production due to the hydrolysis of ATP (●), 2-azido-ATP added in aqueous solution (■) and in methanol (□). In all three cases, 100% phosphate production corresponds to 450 nmol of added nucleotide incubated with 0.4 mg F_1-ATPase. In the case of 8-azido-ATP (▲), 1250 nmol of nucleotide were added to the same amount of F_1. F_1 was pretreated with a gel filtration (three times) on a Penefsky column to remove medium and loosely bound nucleotides. F_1 was dissolved in medium B supplemented with 10 mM HCO_3^- (pH 8.0). Values of phosphate produced were corrected for contaminating phosphate present in the nucleotide solutions. Incubation was performed at room temperature. The reactions were started by the addition of substrate.

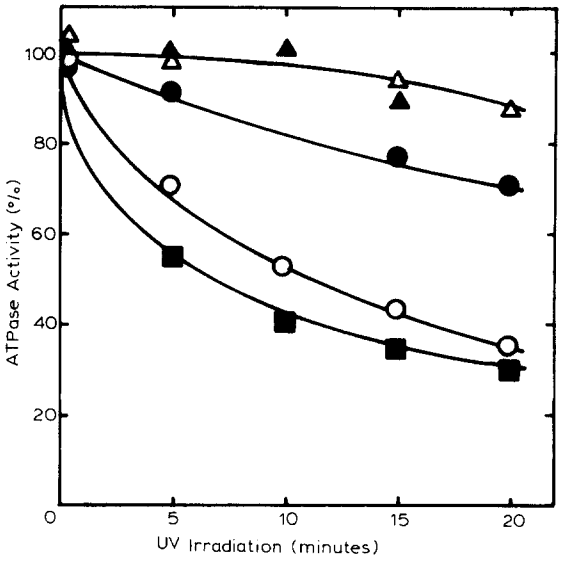

Fig. 3. Time-dependent photo inactivation of F_1 with different photoaffinity labels: 2-azido-ATP (●), 2-azido-ADP (○), 8-azido-ATP (■). The label concentration used was 500 μM. F_1 was dissolved in medium A except in the case of 2-azido-ADP (medium B). Addition of ATP (500 μM) (△) or ADP (950 μM) (▲) to 2-azido-ATP could avoid photoinactivation. In all cases, the remaining activity was compared to irradiated F_1 in the absence of photolabel.

duced approached the amount of nucleotides added. In the same figure, it can be seen that 8-azido-ATP was hydrolysed very slowly: its initial rate of phosphate production was about 3% of that of ATP.

Photo inactivation

The use of 2-azido-ATP as a photoreactive ligand is shown in Fig. 3: both 2-azido-ATP and 2-azido-ADP could be used to inactivate F_1. The inactivation in the presence of Mg^{2+} and 2-azido-ADP exceeded the inactivation in the presence of EDTA and 2-azido-ATP. The inactivation by the 2-azido nucleotides still continued after 20 min of irradiation, whereas in the case of the 8-azido-ATP the inactivation had by then almost stopped. Presumably, this can also be attributed to the slow conversion of the tetrazolic isomers (which are not sensitive to light) to the azido-form. The photo inactivation with the 2-azido analogues was studied in more detail by competition experiments in the presence of additional ATP and ADP. In Fig. 3, it can be seen that in the presence of 0.5 mM ATP as well as 0.95 mM ADP photoinactivation could be avoided, indicating a competition for (catalytic) nucleotide binding sites on the enzyme.

High-affinity binding on catalytic sites

One of the outstanding features of the 2-azido analogues compared to the 8-azido analogues proved to be the capacity of tight binding to the F_1-ATPase. This is illustrated in Fig. 4, where it is shown that incubation of F_1 (dissolved in medium A and depleted only of loosely bound nucleotides) with 2-azido-ATP followed by a gel filtration executed three times on a Penefsky column (to remove medium and loosely bound nucleotides) resulted in extensive inactivation of the enzyme after illumination. It can be assumed that the high-affinity binding of the 2-azido-ATP occurred

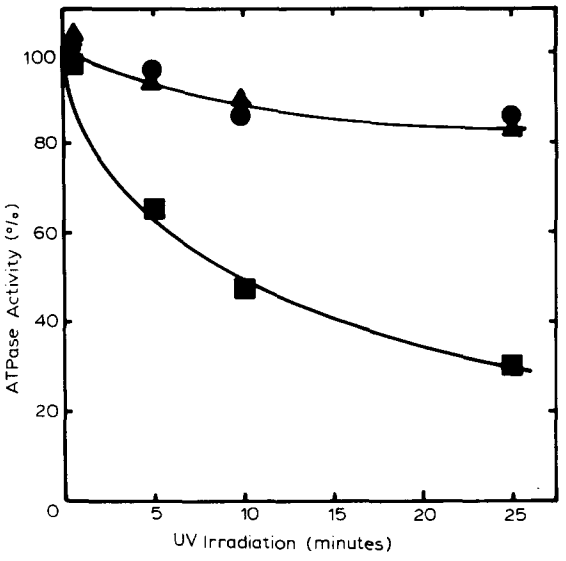

Fig. 4. Demonstration of high-affinity binding of 2-azido-ATP. Isolated F_1 was incubated for 60 s with 6.0 mM of the following nucleotides: ATP (●), 8-azido-ATP (▲) and 2-azido-ATP (■) in medium A. After this incubation, loosely bound and medium nucleotides were removed by a gel filtration executed three times on a Penefsky column with Sephadex pre-equilibrated in medium A. Subsequently, F_1 was irradiated and the hydrolysis activity was measured at various time intervals.

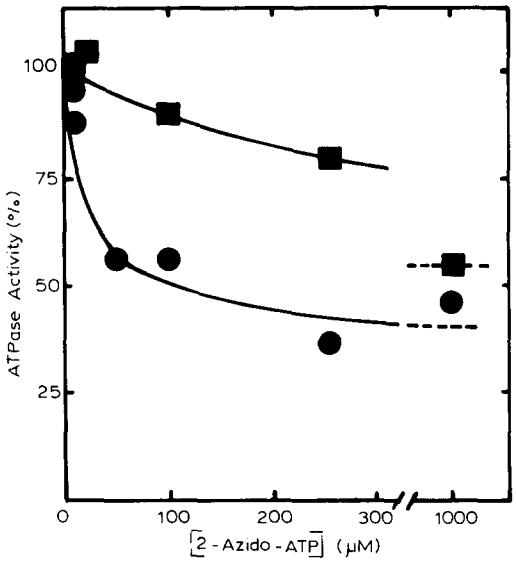

Fig. 5. Concentration dependency of tight incorporation of 2-azido-ATP in medium A (■) and in medium B supplemented with 10 mM HCO_3^- (pH 8.0) (●). For experimental conditions see legend to Fig. 4.

in a catalytic site on the enzyme, because illumination resulted in inactivation. This tight binding occurred only with the 2-azido-nucleotide, because incubation in the presence of 8-azido-ATP did not result in inactivation when illumination was preceded by removal of loosely bound nucleotides (Fig. 4). A similar experiment, in which [2-^{3}H]8-azido-ATP was used did not result in covalent attachment of this label to F_1, indicating that the 8-azido-ATP was not tightly bound at all under these conditions (results not shown).

The tight binding of 2-azido-nucleotides has been studied in more detail. Fig. 5 shows that after incubation of F_1 at high concentrations of 2-azido-ATP (1 mM) in medium A (+EDTA) as well as in medium B (+$MgCl_2$) and the subsequent removal of loosely bound nucleotides, the analogue remained tightly bound to the enzyme. At lower ligand concentrations, however, the tight incorporation occurred only in the presence of Mg^{2+}. The incorporation is expressed as the extent of inactivation obtained after ultraviolet illumination of the incorporated analogue under standard conditions. The results of the experiments represented in Fig. 5 suggest that for tight incorporation, turnover of the enzyme or the presence of Mg^{2+} is required. The time-dependency of the incorporation is illustrated in Fig. 6. In the presence of 250 μM 2-azido-AT(D)P and Mg^{2+} the incorporation occurred within a few seconds. In EDTA-containing medium the rate of incorporation of the nucleotide analogues was much slower, with $t_{1/2}$ values of about 8 s for the incorporation at 250 μM 2-azido-AT(D)P. Both 2-azido-ATP and 2-azido-ADP were substrates for the incorporation with high affinity into a catalytic site, indicating that no turnover is required, but the presence of Mg^{2+} is.

An integrated experiment in which not only the inactivation was measured upon illumination after the removal of non-tightly bound nucleotides, but also the phosphate production due to the hydrolysis of 2-azido-ATP and 8-azido-ATP was followed, is shown in Fig. 7. Firstly, it can be seen that in the case of 8-azido-ATP the initial rate of phosphate production was, indeed, slower than that in the case of 2-azido-ATP. Secondly, only a minor incorporation of 8-azido-ATP into a cata-

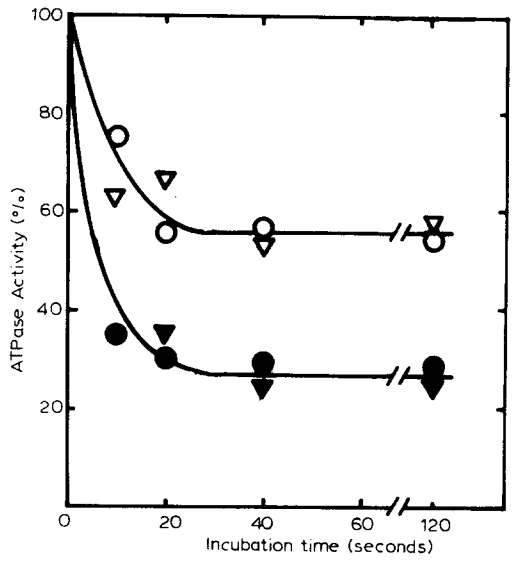

Fig. 6. Time dependency of tight incorporation of 250 μM 2-azido-ATP and 2-azido-ADP in medium A and in medium B (with 10 mM HCO_3^- (pH 8.0)). 2-azido-ATP (▼) and 2-azido-ADP (●) in the adapted medium B, 2-azido-ATP (▽) and 2-azido-ADP (○) in medium A. For experimental conditions see legend to Fig. 4.

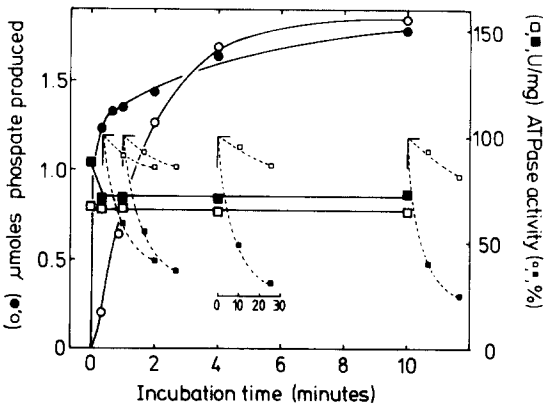

Fig. 7. Phosphate production due to the hydrolysis of 8-azido-ATP (○) or 2-azido-ATP (●) by F_1 dissolved in medium B (supplemented with HCO_3^-, (pH 8.0)). At the indicated times, samples were drawn to measure the amount of phosphate produced. At the same time samples were drawn, filtered three times on a Penefsky column (see legend to Fig. 4) and subsequently tested for ATPase activity, the activity of the F_1 preincubated with 2-azido-ATP (■) or with 8-azido-ATP (□). Subsequently, these samples were irradiated for 10 and 25 min (see inserted scale) and tested for their ATPase activity. The small squares represent the relative inactivation of the irradiated samples; open symbols represent F_1 preincubated with 8-azido-ATP, filled-in symbols represent F_1 preincubated with 2-azido-ATP.

lytic site was found, even after 10 min of incubation, whereas the 2-azido analogue was rapidly incorporated.

The extent of inactivation by covalent binding upon illumination of already tightly bound 2-azido-ATP increased slowly during the experiment. In Fig. 7, and also in Fig. 4, it can be seen that prolonged ultraviolet irradiation resulted in a strongly inactivated enzyme, indicating that the tetrazolium forms of the analogue are not tightly bound or undergo (rapid) conversion to the azido form.

When [β-^{32}P]2-azido-AT(D)P is used in the incorporation experiments, the amount of covalently bound analogue needed for complete inactivation can be determined. After measuring the hydrolysis activity, the amount of covalently bound [β-^{32}P]2-N-AT(D)P was measured by counting the radioactivity of the pellet obtained after heat denaturation and subsequent centrifugation (Fig. 8). A linear relationship was found between the extent of inactivation and the bound label, extrapolating to 0.92 mol label per mol F_1 at full inactivation of the enzyme. It can be concluded that the covalent attachment of only 1 mol [β-^{32}P]2-N-ATP is required for the complete inactivation of the enzyme.

More than 90% of the radioactivity was found on the β subunits as determined via urea-SDS polyacrylamide gel electrophoresis (Fig. 9). This latter result was also found when F_1 was irradiated in the presence of 400 μM [β-^{32}P]2-azido-AT(D)P, although, then, up to about 4 mol of label were bound (Fig. 8). Under the latter conditions a non-linear relationship was found between binding and inhibition indicating that more than one catalytic site is involved, in contrast to the results obtained with [2-^{3}H]8-azido-ATP [7].

Binding of 2-azido-AT(D)P to non-exchangeable sites on F_1

It has been reported previously [2,7,10,18] that 8-azido-AT(D)P can be used to label F_1 to a maximum of 4 mol label per mol F_1, independent of the presence of the so-called non-exchangeable tightly bound nucleotides [11,12]. It has been

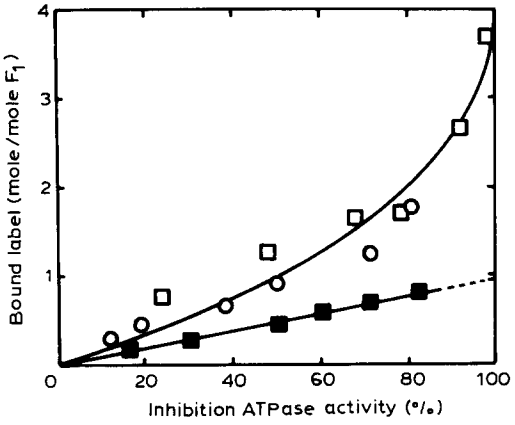

Fig. 8. The relationship between the extent of inactivation of ATPase activity and the amount of bound [β-^{32}P]2-N-AT(D)P (mol/mol F_1). The amounts of covalently bound [β-^{32}P]2-N-AT(D)P were determined by counting the radioactivity of the pellet obtained after heat denaturation and subsequent centrifugation. Heat denaturation of F_1 was performed in the presence (○, □) and absence (■) of bovine serum albumin (3 mg/ml). Irradiation was performed with F_1 containing incorporated [β-^{32}P]2-azido-ATP (■) (obtained after 5 min of incubation in the presence of 500 μM label in medium B and subsequent column centrifugation (three times)). F_1 was also irradiated in the presence of 400 μM [β-^{32}P]2-azido-ATP in medium A (○) or [β-^{32}P]2-azido-ADP in medium B (□). In all cases, samples were drawn at various time intervals to measure the hydrolysis activity and amount of covalently bound photolabel. The extent of inactivation was determined by comparing the hydrolysis activity of F_1 irradiated in the presence of photolabel to the activity of F_1 to which similar amounts of ATP or ADP were added.

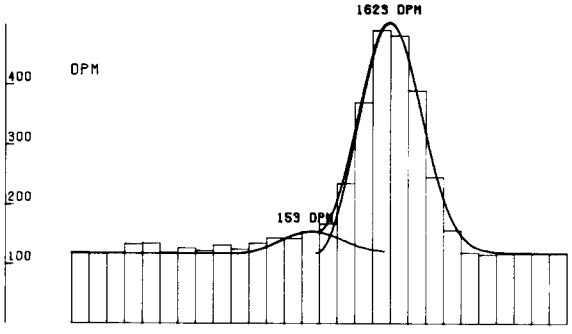

Fig. 9. Distribution of radioactivity over α and β subunits after labelling of F_1 with 250 μM [β-^{32}P]2-azido-ADP in medium B. A sample of 27 μg of the labelled F_1 was applied to the SDS-urea gel and electrophoresed. Bars represent the amount of radioactivity extracted from gel slices in the α/β region. A computer analysis [7] was used to determine the distribution over α and β subunits. The two peaks of radioactivity corresponded with the A_{560} scan of the stained gel (not shown).

shown previously [7] that these two so-called non-exchangeable tightly bound nucleotides are still present on the F_1 after covalent binding of about 4 mol of the 8-azido-AT(D)P to F_1, but they can subsequently be removed by using 0.8 M of LiCl to about 0.8 mol of nucleotides per mol of F_1. Subsequent labelling of F_1 with [2-^{3}H]8-azido-AT(D)P resulted only in minor extra labelling. However, if [β-^{32}P]2-azido-ATP was used instead, at least one site was labelled whereby most radioactivity was found on the β subunits [7]. In this case, a total of about six nucleotides could be calculated to be present on F_1, reflecting the most probable total number of nucleotide binding sites on the enzyme.

Discussion

In Table I it can be seen that 2-azido-ATP is a good substrate analogue for F_1. Due to the presence of tautomeric forms of the analogue, the real K_m value is 2.2-times lower than the apparent value and is comparable to the value for ATP. The V_{max} values for ATP and 2-azido-ATP hydrolysis are of the same order of magnitude (Table I). Furthermore, the steady-state hydrolysis of 2-azido-ATP showed the same non-linear relationship in a Lineweaver-Burk plot as that of ATP in the absence of an activating anion. This indicates that the hydrolysis of the analogue is subject to a similar regulation as is ATP, assuming that this non-linearity is caused by the binding of nucleotide (analogue) to a regulatory site [29,30], or that it is hydrolysed by the same number of catalytic sites, assuming that the non-linearity is due to the involvement of a third catalytic site [15]. The presence of exchangeable non-catalytic binding sites on F_1 was demonstrated with [2-^{3}H]8-azido-AT(D)P [7,10,18], this type of binding site being located at the interface between α and β subunits. Labelling experiments with [β-^{32}P]2-azido-AT(D)P resulted in all cases in the binding of more than 90% of the radioactivity to β subunits. This has been found for the whole concentration range used in the Lineweaver-Burk plot (Fig. 1). It is likely, then, that also the labelling of non-catalytic (interface) sites results in covalent attachment of the analogue mainly to the β subunits. The data cannot be interpreted by assuming that

the 2-azido analogue does not bind to the (regulatory?) interface binding sites, as does the 8-azido-analogue. The difference between the two types of analogue in labelling the α and β subunits must be attributed to the different location of the photoreactive azido group in the adenine ring.

In Fig. 2, it can be seen that the phosphate production by 2-azido-ATP follows a biphasic pattern. It is very likely that the slow (second) phase is due to the rate-limiting conversion of tetrazolic forms to the hydrolysable azido form. This notion is supported by the finding that when 2-azido-ATP was dissolved in methanol (in this medium 80% of the analogue is present in the azido form [25]), the initial amount of phosphate produced was indeed 80% of the total 2-azido-ATP added. The kinetic parameters for the hydrolysis of 8-azido-ATP (Table I) show that this analogue is a less easily hydrolysable substrate. A Lineweaver-Burk plot for the steady-state hydrolysis of 8-azido-ATP by isolated F_1 has recently been reported by Sloothaak et al. [26]. The presence of the (non-photoreactive) tetrazolic isomers of 2-azido-AT(D)P was at least partly responsible for the less impressive inactivation of F_1 upon illumination compared to 8-azido-ATP (Fig. 3). The results of the competition experiments between the 2-azido-analogues and AT(D)P are in agreement with those of competition experiments between 8-azido-AT(D)P and AT(D)P [31,32].

Besides the demonstration of the very good kinetic parameters for the hydrolysis of 2-azido-ATP by F_1, it is shown in this paper that the 2-azido analogues have the unique feature of binding with high affinity to catalytic sites on the F_1 (Fig. 4), as has also been demonstrated in CF_1 [35]. In Fig. 4, it can be seen that such tight binding to a catalytic site does not occur with 8-azido-ATP in the presence of EDTA. Possible tight binding to non-catalytic sites (in which case illumination would not result in inactivation) could be excluded on the basis of an experiment with [2-^{3}H]8-azido-ATP in which no label was found on F_1 after repeated Sephadex filtration and illumination. In the presence of Mg^{2+}, small amounts of the 8-azido analogue were not removed by repeated Sephadex filtration (Fig. 7). The capacity of tight binding of the 2-azido analogue has been described to be dependent on its intramolecular 'anti' configuration [19]. This also explains why 'syn' 8-azido-ATP is not tightly bound to F_1 under the conditions in which 2-azido-ATP is. As illumination of F_1 containing incorporated 2-azido-AT(D)P resulted in inactivation, it can be concluded that a high-affinity state during the turnover exists on (one of the) catalytic sites. The experimental finding of a low V_{max} for the hydrolysis of 8-azido-ATP can then be explained by the fact that the 8-azido-ATP is hardly capable (due to its 'syn' configuration) of passing through this high-affinity state. This is supported by the finding that 8-azido-ATP is hardly able to bind tightly to the F_1.

Although inhibition of the F_1-ATPase activity is observed upon illumination of F_1 with incorporated 2-azido-ATP, it cannot be distinguished whether the analogue was only bound in its azido form or (also) in its tetrazolic forms and whether it was bound in such a way that the interconversion still continued. Fig. 2 suggests that it is only the azido form which is hydrolysed.

Especially at lower concentrations of 2-azido-ATP, the tight incorporation seems to be a Mg^{2+}-requiring process (Fig. 5). This is also illustrated by following the time course of the incorporation (Fig. 6). As both 2-azido-ATP and 2-azido-ADP could be tightly bound, it can be concluded that this tight binding does not require real turnover, i.e., dissociation of ADP due to the addition of ATP. In the presence of EDTA, competition for the Mg^{2+} present between the Mg^{2+}-chelating quality of EDTA and the Mg^{2+} binding of the 2-azido-ATP could be responsible for the lowered incorporation. An integrated experiment, represented in Fig. 7, shows both phosphate production due to the hydrolysis of 2-azido-ATP and 8-azido-ATP, and the tight incorporation of the analogues. The minor incorporation of the 8-azido-ATP possibly reflects the lower rate of hydrolysis of this analogue compared to 2-azido-ATP.

When [β-^{32}P]2-azido-ATP was incorporated into a high-affinity site on the F_1, 0.92 mol of label per mol of F_1 were needed for complete inactivation (Fig. 8). It can be concluded that the covalent attachment of only 1 mol of 2-N-ATP per mol of F_1 is required for the complete inactivation of the enzyme. The data of Melese and

Boyer [35] interpreted by the authors as suggestive for the retention of catalytic cooperativity by the two remaining subunits after one subunit has already been labelled with 2-azido-ATP, can easily be explained by the assumption that under their conditions not one specific catalytic site was labelled but several, both catalytic and non-catalytic. Just one centrifugation through a Penefsky column removes only part of the loosely bound 2-azido-ATP. The high-affinity binding of the 2-azido analogues is very likely similar to the tight binding of ADP to catalytic sites, as proposed by Drobinskaya et al. [17].

As this high-affinity binding is to a catalytic site, the catalytic sites involved cooperate in an alternating site or sequential mechanism. These results are in agreement with results found with [2-^{3}H]8-azido-ATP when this label was used at a low concentration (20 μM): also, in that case, only 1 mol of label per mol of F_1 was required for the complete inactivation of the enzyme, and the radioactivity was found on the β subunits only [7,10,18]. At higher concentrations of [2-^{3}H]8-azido-ATP, an α/β interface binding site was colabelled, resulting in a typical 1 : 3 ratio for label bound to α/β [7,10,18]. These results indicate that with [2-^{3}H]8-azido-ATP a catalytic as well as a non-catalytic binding site can be labelled, located on the β subunit and the α/β interface, respectively. This differentiation could not be made with [β-^{32}P]2-azido-AT(D)P, as the radioactivity was mainly (more than 90%) found on the β subunits, also when more than two sites were labelled.

At higher concentrations of [β-^{32}P]2-azido-AT(D)P, without the removal of medium and loosely bound nucleotides, 3–4 mol of label were bound. From this result, it can be concluded that 2-azido-ATP binds to both types of site. This will be investigated further by determining the amino acid residues labelled by the 2-azido analogue, as has been performed for 8-azido-ATP [34]. With this latter photolabel, indeed two different sites were demonstrated on the β subunits. The non-linearity between binding and inactivation can be explained by the assumption that more than one catalytic site was labelled, all causing the inhibition of the enzyme. The shape of the curve corresponds to the theoretical curve for the case in which, among other (non-catalytic) sites, two catalytic sites, both essential for hydrolysis, are being labelled. When using low concentrations of [α-^{32}P]2-azido-ADP (1–20 μM), Boulay et al. [36] found that 2 mol of bound label were needed for inactivation of F_1, the relationship between binding and inhibition being linear. Since at least three sites bind 2-azido-ADP under the conditions used [36], it is likely that the linear extrapolation to 2 is not correct.

Besides the presence of four exchangeable binding sites, F_1 contains at least two so-called non-exchangeable tight nucleotide binding sites [2,3,8,9] which could be partially labelled with [β-^{32}P]2-azido-AT(D)P but not with the 8-azido analogues when the bound nucleotides were first removed by treating F_1 with LiCl [7]. This difference can also be explained by the requirement for an 'anti' configuration of the nucleotide, which is supported by the relatively high substrate specificity of these sites [2]. Although it is theoretically expected that the two so-called nonexchangeable nucleotides are located on a β subunit and an α/β interface, it is not possible to verify this assumption by measuring the distribution of [β-^{32}P]2-azido-AT(D)P over α and β subunits. Determination of the labelled amino acid residues of the β subunit is required. The differentiation between high-affinity binding to catalytic sites on the one hand and the so-called non-exchangeable tight nucleotide binding sites on the other is under current investigation in our laboratory, as both types of binding seem to require the intramolecular 'anti' configuration of the binding nucleotide.

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References

1 Vignais, P.V. and Satre, M. (1984) Mol. Cell Biochem. 60, 33–70

2 Wagenvoord, R.J., Kemp, A. and Slater, E.C. (1980) Biochim. Biophys. Acta 593, 204–211
3 Cross, R.C. and Nalin, C.M. (1982) J. Biol. Chem. 257, 2874–2881
4 Weber, J., Lücken, U. and Schäfer, G. (1985) Eur. J. Biochem. 148, 41–47
5 Cross, R.L., Grubmeyer, C. and Penefsky, H.S. (1982) J. Biol. Chem. 257, 12101–12105
6 O'Neal, C.C. and Boyer, P.D. (1984) J. Biol. Chem. 259, 5761–5767
7 Van Dongen, M.B.M. and Berden, J.A. (1986) Biochim. Biophys. Acta 850, BBA 42021
8 Harris, D.A., Rosing, J., Van de Stadt, R.J. and Slater, E.C. (1973) Biochim. Biophys. Acta 314, 149–153
9 Slater, E.C., Kemp, A., Van der Kraan, I., Muller, J.L.M., Roveri, O.A., Verschoor, G.J., Wagenvoord, R.J. and Wielders, J.P.M. (1979) FEBS Lett. 103, 7–11
10 Van Dongen, M.B.M., Berden, J.A., Hartog, A.F. and Slater, E.C. (1985) 13th International Congress of Biochemistry, Amsterdam, BBA Abstracts, p. 568, Elsevier Science Publishers, Amsterdam
11 Harris, D.A. and Baltscheffsky, M. (1979) Biochem. Biophys. Res. Commun. 86, 1248–1255
12 Rosing, J., Harris, D.A., Slater, E.C. and Kemp, A. (1975) J. Supramol. Struct. 3, 284–296
13 Grubmeyer, C., Cross, R.L. and Penefsky, H.S. (1982) J. Biol. Chem. 257, 12092–12100
14 Grubmeyer, C. and Penefsky, H.S. (1981) J. Biol. Chem. 256, 3728–3734
15 Gresser, M.J., Myers, J.A. and Boyer, P.D. (1982) J. Biol. Chem. 257, 12030–12038
16 Czarnecki, J.J., Abbott, M.S. and Selman, B.R. (1983) Eur. J. Biochem. 136, 19–24
17 Drobinskaya, I.Ye., Kozlov, I.A., Murataliev, M.B. and Vulfson, E.N. (1985) FEBS Lett. 182, 419–424
18 Berden, J.A., Van Dongen, M.B.M., Sloothaak, J.B. and Hartog, A.F. (1985) in Achievements and Perspectives of Mitochondrial Research (Quagliariello, E., Slater, E.C., Palmieri, F., Saccone, C. and Kroon, A.M., eds.), Vol. I, pp. 257–266, Elsevier Science Publishers, Amsterdam
19 Czarnecki, J.J., Abbott, M.S. and Selman, B.R. (1982) Proc. Natl. Acad. Sci. USA 79, 7744–7748
20 Knowles, A.F. and Penefsky, H.S. (1972) J. Biol. Chem. 247, 6624–6630
21 Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899
22 Schaefer, H.J. and Thomas, H.J. (1958) J. Am. Chem. Soc. 80, 3738–3742
23 Sowa, T. and Ouchi, S. (1975) Bull. Chem. Soc. Jap. 48, 2084–2090
24 Hoard, D.E. and Ott, D.G. (1965) J. Am. Chem. Soc. 87, 1785–1788
25 Czarnecki, J.J. (1984) Biochim. Biophys. Acta 800, 41–51
26 Sloothaak, J.B., Berden, J.A., Herweijer, M.A. and Kemp, A. (1985) Biochim. Biophys. Acta 809, 27–38
27 Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375–400
28 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
29 Recktenwald, D. and Hess, B. (1977) FEBS Lett. 76, 25–28
30 Stutterheim, E., Henneke, M.A.C. and Berden, J.A. (1980) Biochim. Biophys. Acta 592, 415–430
31 Wagenvoord, R.J., Van der Kraan, I. and Kemp, A. (1977) Biochim. Biophys. Acta 460, 17–24
32 Wagenvoord, R.J., Van der Kraan, I. and Kemp, A. (1979) Biochim. Biophys. Acta 548, 85–95
33 Abbott, M.S., Czarnecki, J.J. and Selman, B.R. (1984) J. Biol. Chem. 259, 12271–12278
34 Hollemans, M., Runswick, M.J., Fearnly, I.M. and Walker, J.E. (1983) J. Biol. Chem. 258, 9307–9313
35 Melese, T. and Boyer, P.D. (1985) J. Biol. Chem. 260, 15398–15401
36 Boulay, F., Dalbon, P. and Vignais, P.V. (1985) Biochem. 24, 7372–7379